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13. ABSTRACT ***(Maximum 200 Words)***

Cyr61, a member of the CCN gene family, was isolated and identified by differential expression between estrogen receptor (ER)-positive and ER-negative breast cancer cells. Cyr61 is a ligand for the integrin $\alpha v\beta 3$, which is involved in tumorigenesis and angiogenesis (formation of new blood vessels). We showed previously that expression of Cyr61 in HRG-transfected MCF-7 cells is greatly increased compared to parental MCF-7 cells. We also showed that Cyr61 is expressed in all the invasive, metastatic, HRG-expressing, and ER-negative breast cancer cell lines. Moreover, Cyr61 was detected in about 30% of invasive human breast tumor biopsies. Most significantly, an anti-Cyr61 blocking antibody abolishes the invasiveness and migration of HRG-expressing breast cancer cells *in vitro*. To understand the role of Cyr61 in breast cancer progression, the human Cyr61 cDNA was introduced to ER-negative, HRG-negative breast cancer cells. Cyr61-expressing cells showed a growth advantage in serum-depleted conditions. The preliminary results suggest that Cyr61 is sufficient to promote estrogen independence and anti-estrogen resistance of breast cancer cells both *in vitro* and *in vivo*. Cyr61-expressing breast cancer cells are invasive *in vitro* and tumorigenic *in vivo*. The mechanism involved in Cyr61-induced tumor formation and aggressiveness is mediated via specific activation of the MAPK and PI3K/AKT pathways. Blocking of Cyr61 expression in aggressive breast cancer cells by specific anti-sense oligonucleotides or by constitutive expression of anti-sense Cyr61 messages results in phenotypic reversion, including reacquisition of E2 requirement for growth and anti-E2 sensitivity, in addition to reduced invasiveness and matrix protease activity *in vitro*, and decreased tumoriginecity. Taken together, these results strongly indicate that Cyr61 plays a critical role in breast cancer progression.

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Introduction

Aggressiveness of breast cancer cells is commonly attributed to the ability of these cells to overcome the estrogen (E2) requirements for growth, and in most cases to acquire antiestrogen (anti-E2) resistance. The mechanism by which breast cancer appears to progress from an E2-dependent to an E2-independent phenotype is still under investigation. We have shown that expression of a growth factor, heregulin (HRG), which is an activator of *erbB*-2/3/4 receptor signaling pathways, is closely associated with an invasive breast cancer phenotype (1). Furthermore, we demonstrated that HRG induces breast cancer progression, as determined by loss of estrogen receptor (ER) function and response, tumorigenicity, invasion, and metastasis (2-4). It is possible that HRG induces activation of the *erbB* signaling pathways, leading to regulation of downstream genes that regulate and control cancer progression.

With this in mind, we isolated and identified the human homologue of a mouse immediate-early response gene, Cyr61, differentially expressed in ER-negative, HRG-positive breast cancer cells (5). Cyr61 is a secreted cysteine-rich protein that is associated with the cell surface and the extracellular matrix (ECM) (6, 7). Cyr61 mediates cell adhesion, migration, and angiogenesis (6-10). We have shown that Cyr61 is co-expressed with HRG in all the metastatic breast cancer cell lines tested, its expression is inversely correlated with ER expression, and it is associated with HRG-induced breast cancer chemomigration and metastasis, possibly through interactions with the $\alpha v \beta 3$ integrin receptor (5). Furthermore, we have established that Cyr61 was expressed in about 30% of invasive breast cancer tumor biopsies, implying a possible role of Cyr61 in breast cancer progression (5). The goal of the proposed studies is to determine the involvement of Cyr61 in breast cancer progression. The trainee plans to test whether expression of Cyr61 is necessary and/or sufficient for HRG-induction of breast cancer progression, whether blocking of Cyr61 expression reverts aggressive phenotypes of breast cancer, and whether modulation of Cyr61 expression affects integrin signaling, leading to the acquisition of breast cancer progression.

During the first year of the proposed study, the trainee has generated a constitutive expression vector for the human Cyr61 cDNA and successfully established several stable breast cancer cell lines over-expressing Cyr61. Both *in vitro* and *in vivo* characterization of phenotypic changes in breast cancer cells were initiated and has completed in the second year. On the other

hand, in the previous period, a constitutive expression vector for anti-sense Cyr61 was also generated. In the following year, this vector was introduced to HRG-overexpressing breast cancer cells, and both the *in vitro* and *in vivo* analyses in breast cancer cells were initiated to determine whether blocking Cyr61 expression results in phenotypic reversion.

Please note that the trainee accepted a job offer elsewhere, effective in April 2001. The progress report herein contains results of the proposed studies accomplished between August 1, 2000 and March 23, 2001, that is, in months 13-20.

Body

Task 1 in the proposed study is to determine whether expression of Cyr61 is necessary and/or sufficient to confer E2-independent and anti-E2 resistant phenotypes in ER-positive breast epithelial cells in months 1-22. In the first 12 months, a constitutive expression vector of Cyr61 was constructed and introduced to the MCF-7 cell line. Stable clones were established and expression of Cyr61 was confirmed by RNase protection assays (see the progress report in year 2000). Expression of the Cyr61 protein in the stable lines was further confirmed by collecting the conditioned media, concentrated, and analyzed by Western blotting using a polyclonal antibody specifically against Cyr61. Expression of the Cyr61 protein was consistent with that of its mRNA levels (Figure 1). As expected, low levels of Cyr61 expression were detected in vector cells, similar to those in the wild-type MCF-7 cells. Most of the Cyr61 clones show similar expression level of Cyr61 to MDA-MB-231 cells, an aggressive breast cancer cell line that naturally express high levels of Cyr61.

In vitro characterization of Cyr61 clones was initiated in months 9-12, continued and completed in months 13-16. To determine whether Cyr61 promotes an invasive phenotype, matrigel outgrowth assays were performed. As shown in Figure 2, MCF-7/Cyr61 clones showed extensive outgrowth in Matrigel in the absence of E2; the colonies appeared large and irregular in shape. In contrast, MCF-7/V cells were not able to migrate through and proliferate in Matrigel matrix even in the presence of E2.

It has shown previously that a possible mechanism to acquire E2-independence and anti-E2 resistance is to lose ER expression and/or function. ER function can be measured as the ability of E2 to regulate E2-responsive genes. First of all, the basal levels of ER expression in

MCF-7/V cells were compared with those in MCF-7/Cyr61. As shown in Figure 3, the basal level of ER in MCF-7/Cyr61 clones was markedly reduced (30-50%) as compared with MCF-7/V cells. However, E2 treatments still regulate the expression of E2-responsive genes, such as PgR, Cathepsin D, and pS2, in MCF-7/Cyr61 clones. These results indicate that Cyr61 is sufficient to confer E2 independence and anti-E2 resistance by down-regulating ER expression but not disrupting ER function.

MAPK has been shown activated by integrin-mediated signaling transduction upon cell adhesion to matrix proteins such as collagen, fibronectin, vitronectin, and laminin. To address whether the aggressive phenotypes of breast cancer cells induced by Cyr61 are mediated via this signaling pathway or not, MAPK expression and activation were examined. As shown in Figure 4A, an increased in activated, phosphorylated MAPK (p42/p44) was observed in all of the MCF-7/Cyr61 clones as compared with MCF-7/V cells. The protein expression level of MAPK was also elevated in MCF-7/Cyr61 clones. PI3 kinase has been implicated in cellular migration and motility, where Cyr61 has been shown to be a potentiator. As indicated in Figure 4B, a 25% to 60% increase in activated PI3K was seen in MCF-7/Cyr61 clones. It has been reported that GTP-bound Ras, in addition to binding to Raf, may bind to PI3K, leading to stimulation of AKT. A marked increase in AKT activity was observed in all of the MCF-7/Cyr61 clones as shown in Figure 4C. However, there is no change in AKT protein level among MCF-7/Cyr61 clones and MCF-7/V cells. These results provide substantial evidence for the mechanisms of Cyr61 action. Cyr61 may induce activated Ras signaling (MAPK activity), which may bind and activate PI3K, which in turns stimulates AKT activity. Thus, MAPK and AKT pathways may play an important role in Cyr61-induced tumorigenesis.

Matrix metalloproteases (MMPs) are a group of enzymes involved in matrix degradation, which is an essential step in tumor invasion and metastasis. As shown previously, Cyr61 markedly induces the outgrowth of MCF-7 cells in Matrigel matrix, leading to the hypothesis that Cyr61 may assist in matrix degradation. The expression of any members of MMP family was examined. The secreted gelatinase activities from MCF-7/V or MCF-7/Cyr61 cells were analyzed by gelatin reversed zymography. A significant increase in an activity of about 92 kDa was observed in all MCF-7/Cyr61 clones. The size corresponds to the activated Gelatinase B, also known as MMP9. However, low to undetectable MMP9 was observed in MCF-7/V cells (Figure 5A). No significant activity of MMP2, about 72 kDa, was detected in either control cells

or Cyr61 clones. To ensure that the MMP activity regulated by the expression of Cyr61 was indeed MMP9. Two independent MMP9 inhibitors, R94138 and GM6001 were used. Both inhibitors block the activity of MMP9 in zymogram assays. Moreover, both inhibitors also attenuate Cyr61-induced Matrigel outgrowth in a dose-dependent manner, and inhibit anchorage-independent growth of MCF-7/Cyr61 cells.

In months 15-20, four of Cyr61-overexpressing clones and one vector control clone were inoculated in the mammary fat pad of ovariectomized athymic nude mice to examine the tumor formation and metastatic potential in vivo. Tumors were spontaneously formed only in those mice injected with MCF-7/Cyr61 cells. These tumors grew independently of hormonal stimulation (Figure 6A). On the other hand, tumors were observed in mice injected with MCF-7/V cells only in the presence of E2 stimulation. These data demonstrate that overexpression of Cyr61 promotes tumor growth in vivo in the absence of E2. These findings support the in vitro data where MCF-7/Cyr61 acquires a growth advantage in E2-depleted media becoming E2 independent. Immunohistochemical analysis demonstrates that the level of Cyr61 expression was high in tumors developed from MCF-7/Cyr61 cells, but low or undetectable Cyr61 was found in tumors derived from MCF-7/V cells (Figure 6B). Thus, these data demonstrate that Cyr61 expression is maintained *in vivo*, and that the phenotypic changes *in vivo* are mediated through Cyr61 expression. Since the tumors appear highly vascurlarized, VEGF expression in those tumor sections was confirmed by immunohistochemical staining (Figure 6C). These results were consistent with previous reports, showing that Cyr61 induces tumor formation and angiogenesis in gastric adenocarcinoma cell line and in normal breast cells.

<u>Task 2</u> in the proposal is to determine whether blockage of Cyr61 expression restores E2-responsiveness and/or results in loss of metastatic phenotypes of aggressive breast cancer cells in months 10-36. Three different approaches were proposed to block Cyr61 expression: (1) A constitutive expression vector for anti-sense Cyr61 cDNA (pcDNA3.1/zeo/AS-Cyr61) was constructed and introduced to ER-, HRG+, invasive, and metastatic breast cancer cell lines, such as MDA-MB-231, MDA-MB-435, and MCF-7/HRG (clones T2, T7, and T8); (2) The anti-sense Cyr61 oligonucleotides were synthesized and tested in cultures; and (3) Oligonucleotides to generate ribozymes specific to Cyr61 were designed, and ribozymes were constructed yet characterized.

In months 10-20, efforts were focused on the first two approaches. First, within months 10-13, the anti-sense Cyr61 oligonucleotides were designed, synthesized, and tested in invasive and metastatic MCF-7/HRG cells, as well as MCF-7/Cyr61 cells obtained from Task 1. Blockage of Cyr61 expression using anti-sense Cyr61 oligonucleotides results in inhibition of *in vitro* invasiveness as determined by Matrigel outgrowth assays (Figure 7). However, the control, sense Cyr61 oligonucleotides has no effect on the outgrowth pattern formation induced by both cell lines.

The next approach is to generate a constitutive expression vector for anti-sense Cyr61 cDNA. In months 12-14, the anti-sense expression vector (pcDNA3.1/zeo/AS-Cyr61), constructed in months 10-11, was introduced by electroporation into ER^-, HRG^+, invasive, and metastatic breast cancer cell lines MCF-7/HRG clone T7 or MDA-MB-231. Stable lines of anti-sense Cyr61-expressing clones (designated as T7AC or 231AC) and empty vector control clones (designated as T7V or 231V) were established by zeocin selection in months 13-14. Ten vector control clones and ten AS-Cyr61-transefected clones were isolated.

In vitro characterization of AS-Cyr61 transfectants has been initiated during months 15-18. To examine re-acquisition of E2 dependence and anti-estrogen resistance in AS-Cyr61-expressing MCF-7/T7 clones, growth assays in regular media, and anchorage-dependent and anchorage-independent growth assays in E2-depleted conditions were performed. Only data obtained from T7AC clones were presented in this progress report. Similar results were observed in 231AC cells. Cellular proliferation is reduced in anti-sense Cyr61-transfected cells as compared to parental T7 or vector control cells (Figure 8). Moreover, anti-sense Cyr61-transfectants become E2-dependent and anti-E2-sensitive in both anchorage-dependent and anchorage-independent growth assays (Figure 9).

Furthermore, blocking Cyr61 expression promotes cells to lose their ability to invade through Matrigel matrix as determined by Matrigel outgrowth assay (Figure 10). It is clearly demonstrated that the MMP9 activity in AS-Cyr61 clones is significantly reduced to similar levels in MCF-7 wild-type cells (Figure 11).

The current data show that blocking the expression of Cyr61 results in *in vitro* phenotypic reversion of MCF-7/HRG cells from an E2-independent and anti-E2-resistant to E2-dependent and anti-E2-sensitive phenotype, and that down-regulation of Cyr61 inhibits *in vitro* invasiveness by blocking outgrowth in Matrigel matrix and reducing matrix protease activity. ER expression

and ER function will be further determined to explore the possible mechanism involved in re-acquisition of hormone dependence and anti-E2 sensitivity in AS-Cyr61-expressing MCF-7/HRG T7 cells. The possible signaling pathways, including MAPK, PI3K, and AKT pathways will be examined to determine whether inactivation of these signaling molecules occurs due to reduction of Cyr61 expression, and whether these pathways are involved in the phenotypic reversion of aggressive and invasive breast cancer cells.

The initial *in vivo* characterization of AS-Cyr61 cells has been performed in months 18-20. The preliminary data indicated that blocking Cyr61 expression in MCF-7/HRG cells significantly delays the onset of tumor formation and markedly reduces the size of breast tumors (Table 1), which are correlated to the expression level of Cyr61 in breast cancer cells as determined by immunohistochemical analysis. E2 requirement and anti-E2 sensitivity in AS-Cyr61 cells in the *in vivo* nude mice system is currently under investigation.

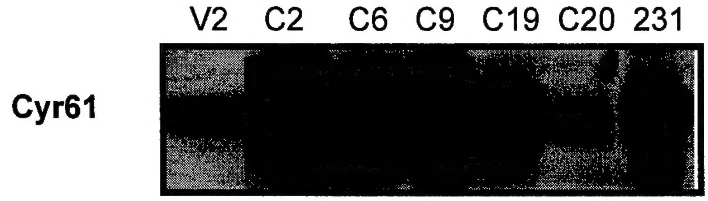


Figure 1. Expression of the Cyr61 protein in MCF-7/Cyr61 clones. Subconfluent MCF-7/Cyr61 clones and MCF-7/V cells were cultured in serum-free media for 48 hr. Conditioned media were collected, concentrated, and analyzed by Western blotting analysis with a rabbit polyclonal anti-Cyr61 antibody. Experiments were repeated at least three times and similar results were obtained.

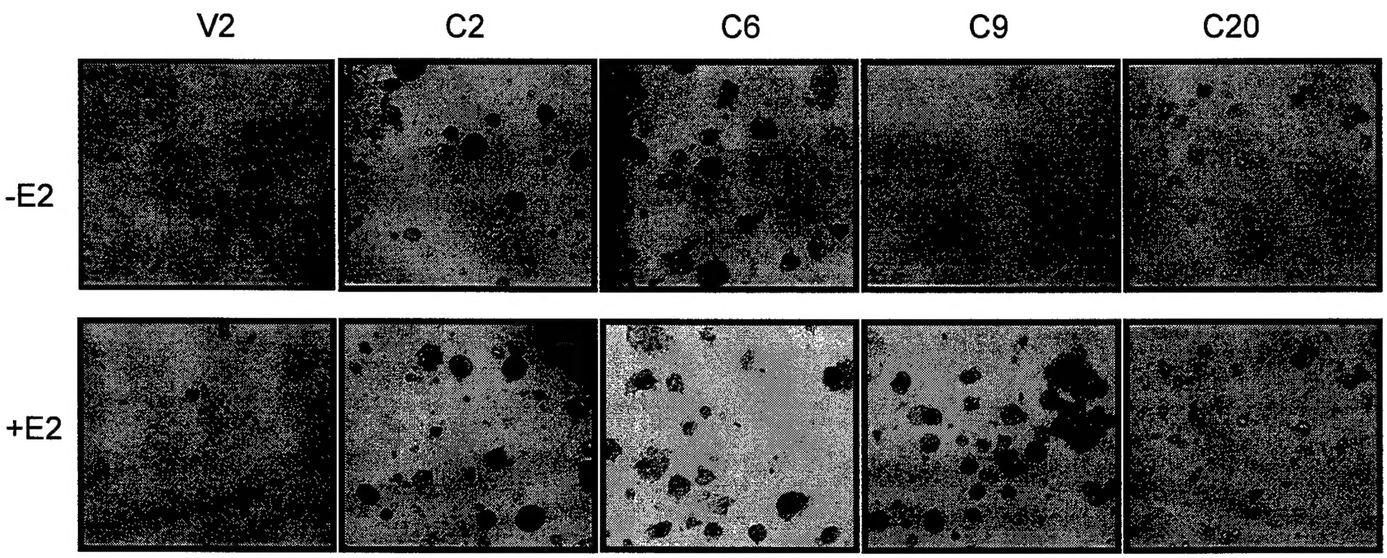


Figure 2. Overexpression of Cyr61 induces outgrowth of MCF-7 cells in Matrigel matrix. Subconfluent MCF-7/V and MCF-7/Cyr61 clones were cultured in E2-depleted media for 4 days and plated in triplicate in the presence or absence of E2 in Matrigel. Outgrowth pattern was developed and photographed after 10-day incubation. Five representative clones are shown with similar results from three independent experiments.

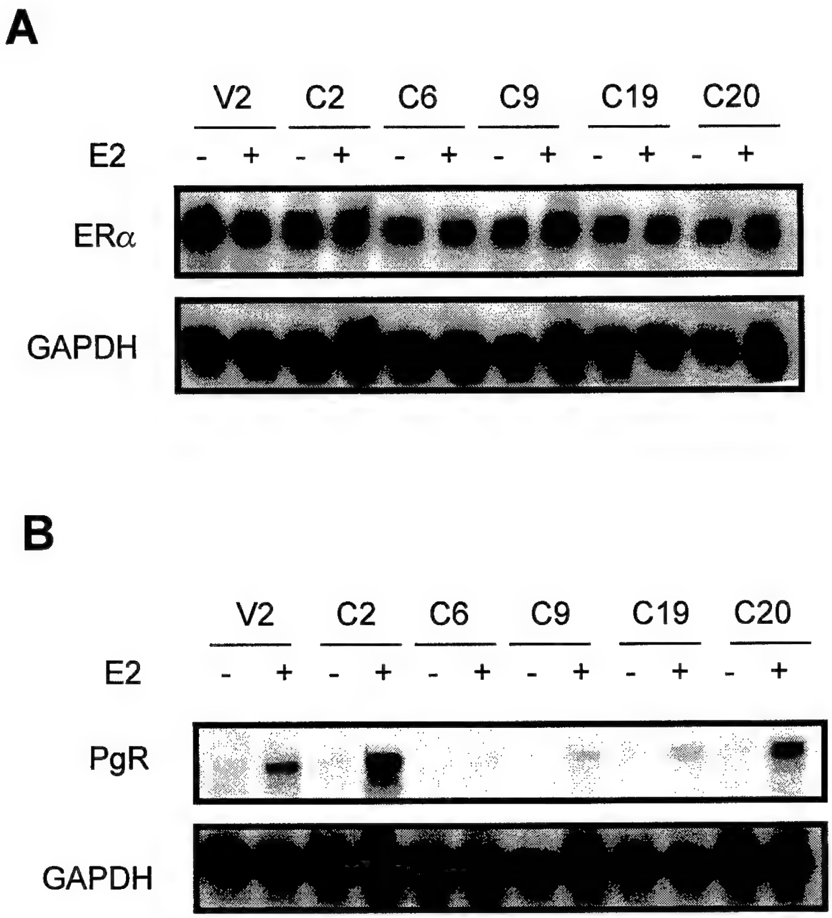


Figure 3. Cyr61 expression does not disrupt E2-responsive gene expression in MCF-7 cells. Subconfluent MCF-7/V and MCF-7/Cyr61 cells were cultured in media containing charcoal-stripped serum for 4 days and treated in the presence or absence of E2 for 6 hrs. Total RNA was isolated and analyzed for the expression of (A) ERα and (B) PgR by the RNase protection assay as described previously. Similar results were obtained from at least three independent experiments. Expression of ERα or PgR was normalized by GAPDH.

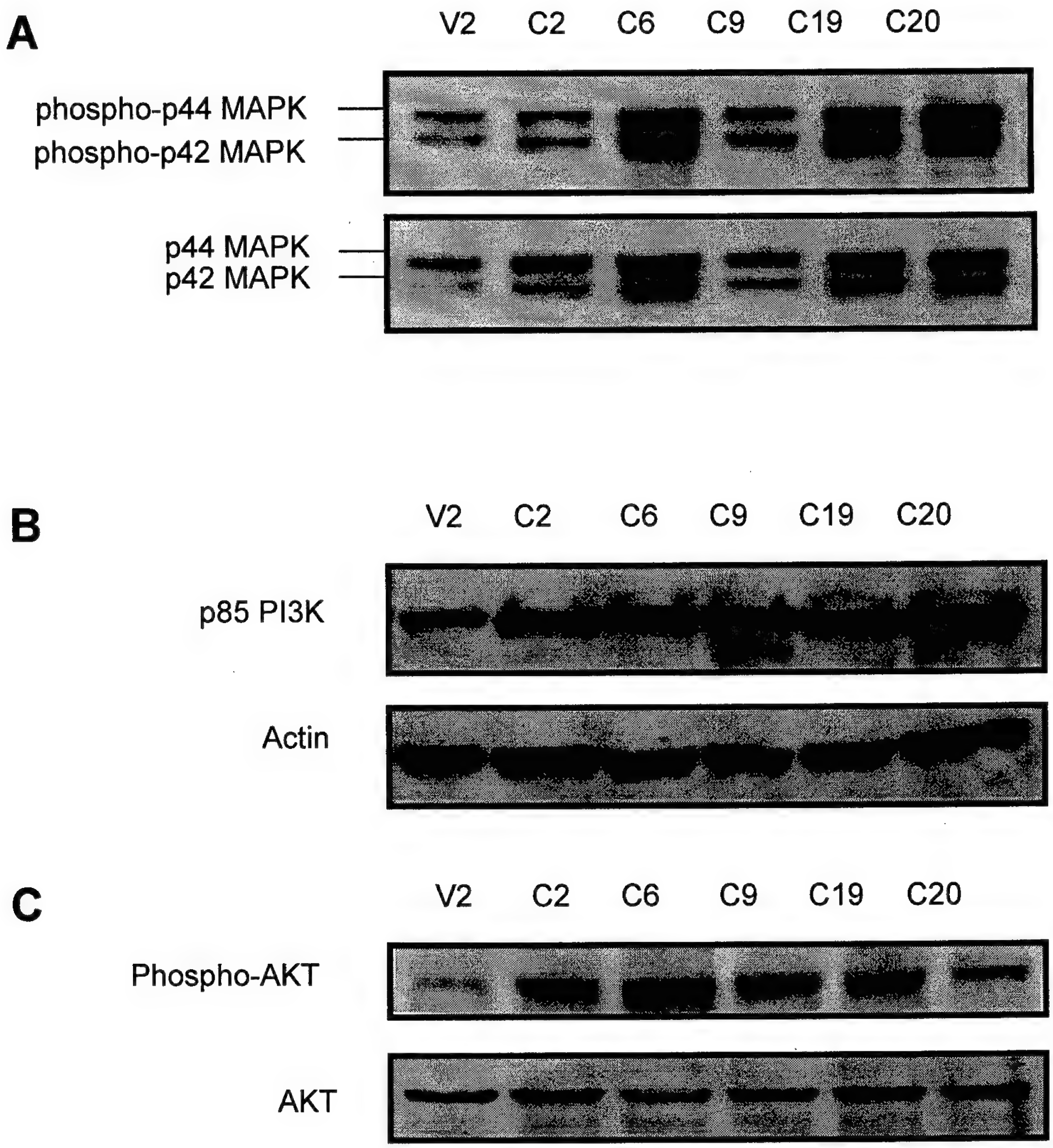
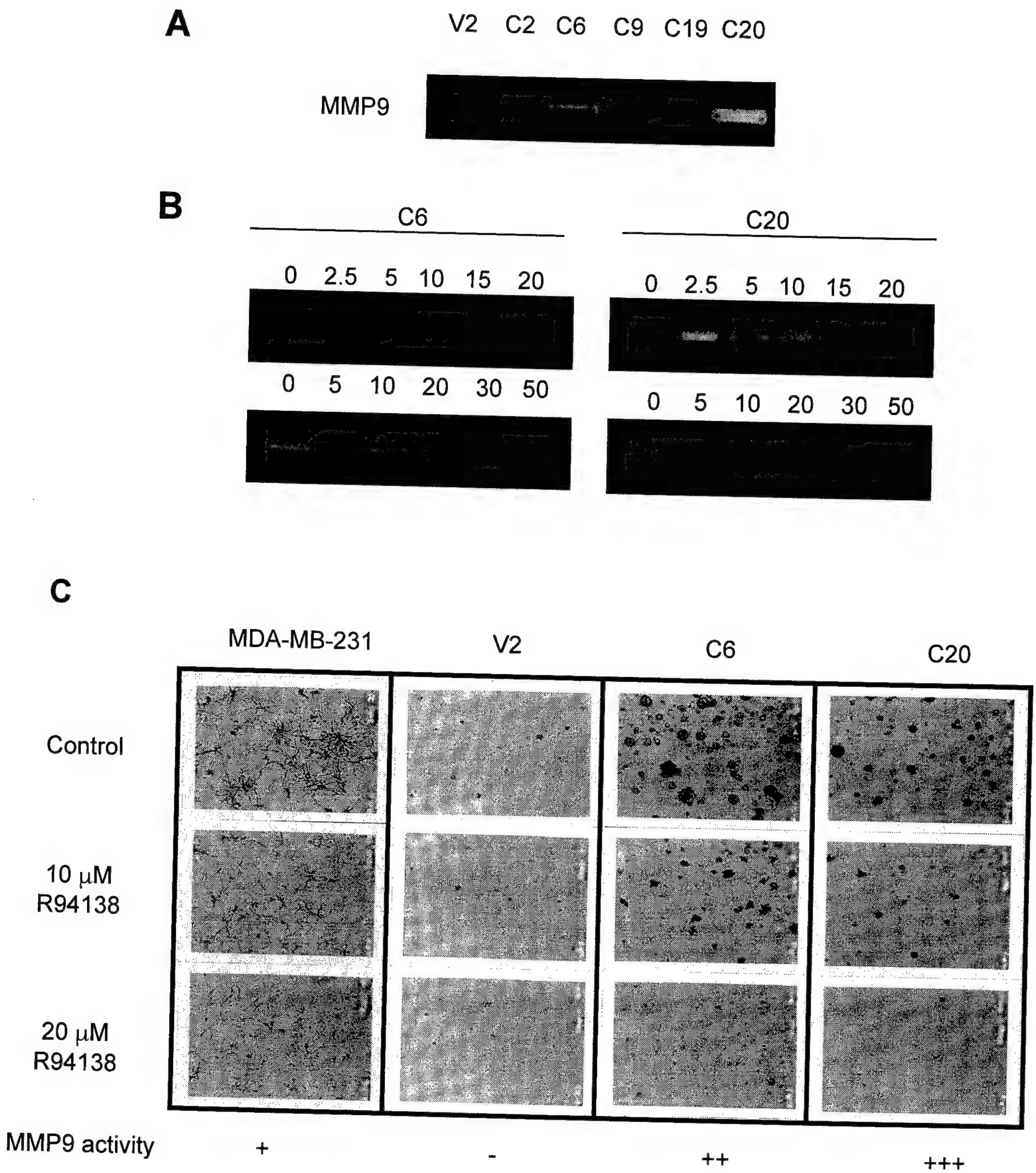


Figure 4. MAPK, PI3K, and AKT are activated in MCF-7/Cyr61 clones. Subconfluent MCF-7/V and MCF-7/Cyr61 cells were serum-starved for 24 hr. Cell lysates were collected, resolved by SDS-PAGE, and immunoblotted with (A) anti-phosphorylated MAPK antibody and anti-MAPK antibody (1:1000); with (B) anti-PI3K p85 antibody (1:1000), and anti-actin antibody (100 μg/ml); and with (C) anti-phosphorylated AKT and anti-AKT antibodies (1:1000).


A
V2 C2 C6 C9 C19 C20
MMP9
B
C6
0 2.5 5 10 15 20
0 5 10 20 30 50
C20
0 2.5 5 10 15 20
0 5 10 20 30 50
C
MDA-MB-231
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+
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D

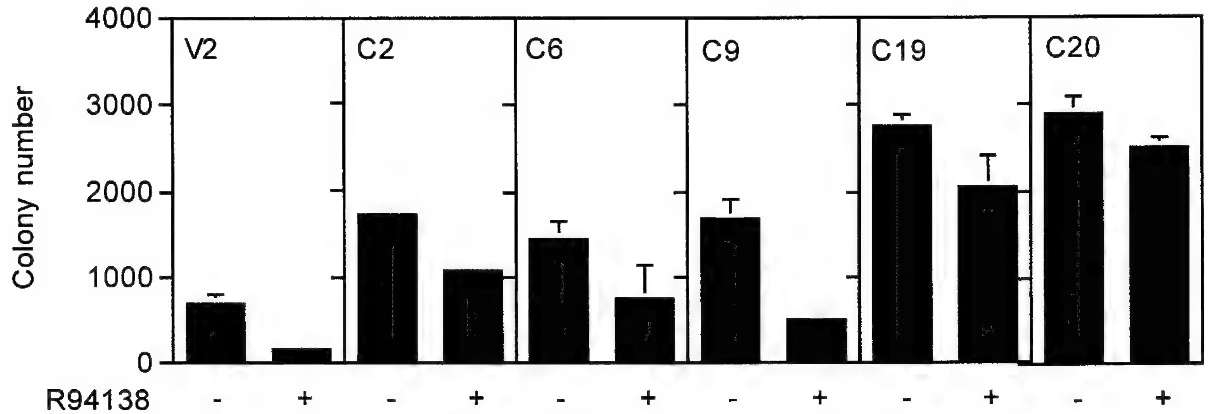
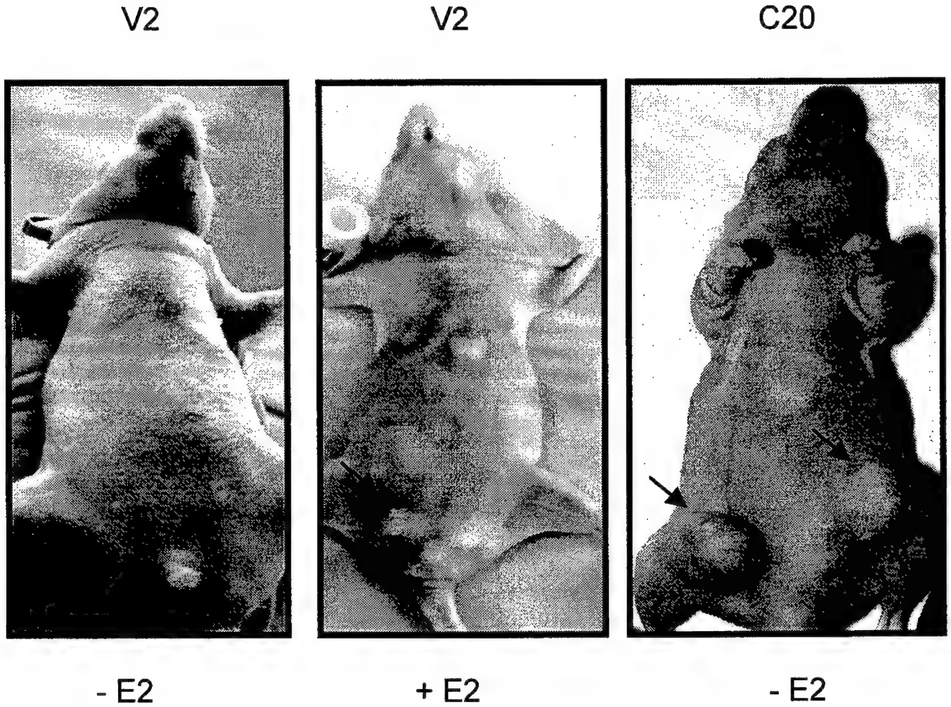


Figure 5. MMP9 activity is increased in MCF-7/Cyr61 clones. (A) Subconfluent MCF-7/V and MCF-7/Cyr61 clones were cultured in serum-free media for 48 hr. Conditioned media were collected, concentrated, and analyzed for MMP9 activity using gelatin zymography. (B) Subconfluent MCF-7/Cyr61 clones were treated with MMP9 inhibitors R94138 (top panel) or GM6001 (bottom panel) at indicated concentrations in serum-free media for 48 hr, and MMP9 activity was analyzed. (C) Subconfluent MCF-7/V and MCF-7/Cyr61 clones were plated in duplicate in Matrigel in the presence or absence of R94138. Outgrowth pattern was developed and photographed as described previously. (D) Subconfluent MCF-7/V and MCF-7/Cyr61 clones were plated in triplicate for soft agar assay in the presence or absence of R94138 (20 μM). Colony number was determined after 12-14 day incubation.

A



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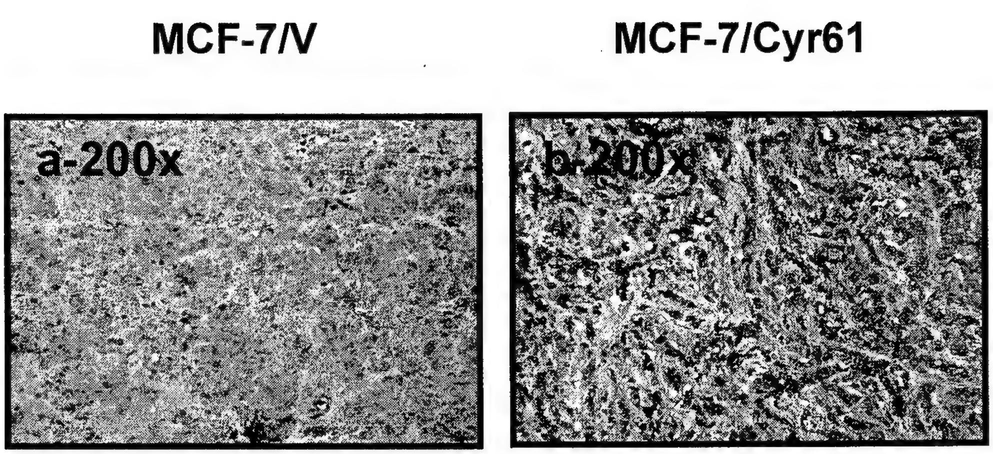

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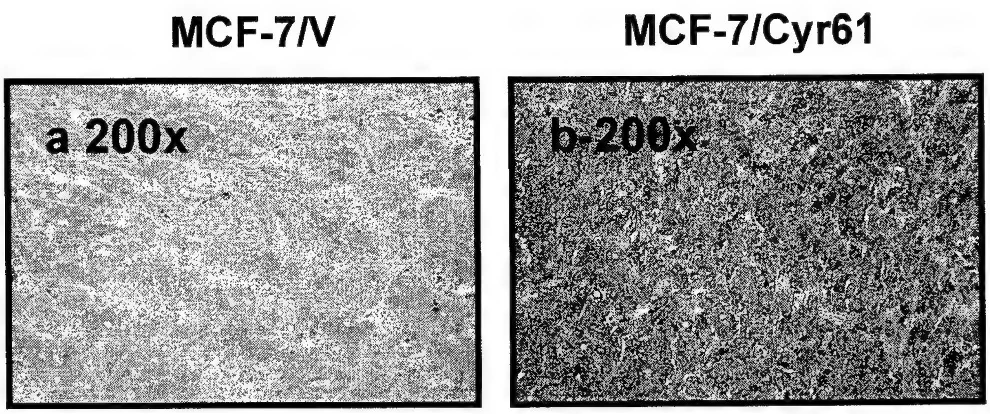

Figure 6. Expression of Cyr61 and VEGF are detected in implanted breast tumor sections from nude mice. (A) Photographs of nude mice bearing human breast tumors in the presence or absence of E2. (B) Immunohistochemical analysis of Cyr61 expression in breast tumor sections developed from implanted MCF-7/V (a-200x) and MCF-7/Cyr61 (b-200x) cells in nude mice. (C) Immunohistochemical analysis of VEGF expression in breast tumor sections derived from implanted MCF-7/V (a-200x) and MCF-7/Cyr61 (b-200x) cells in nude mice.

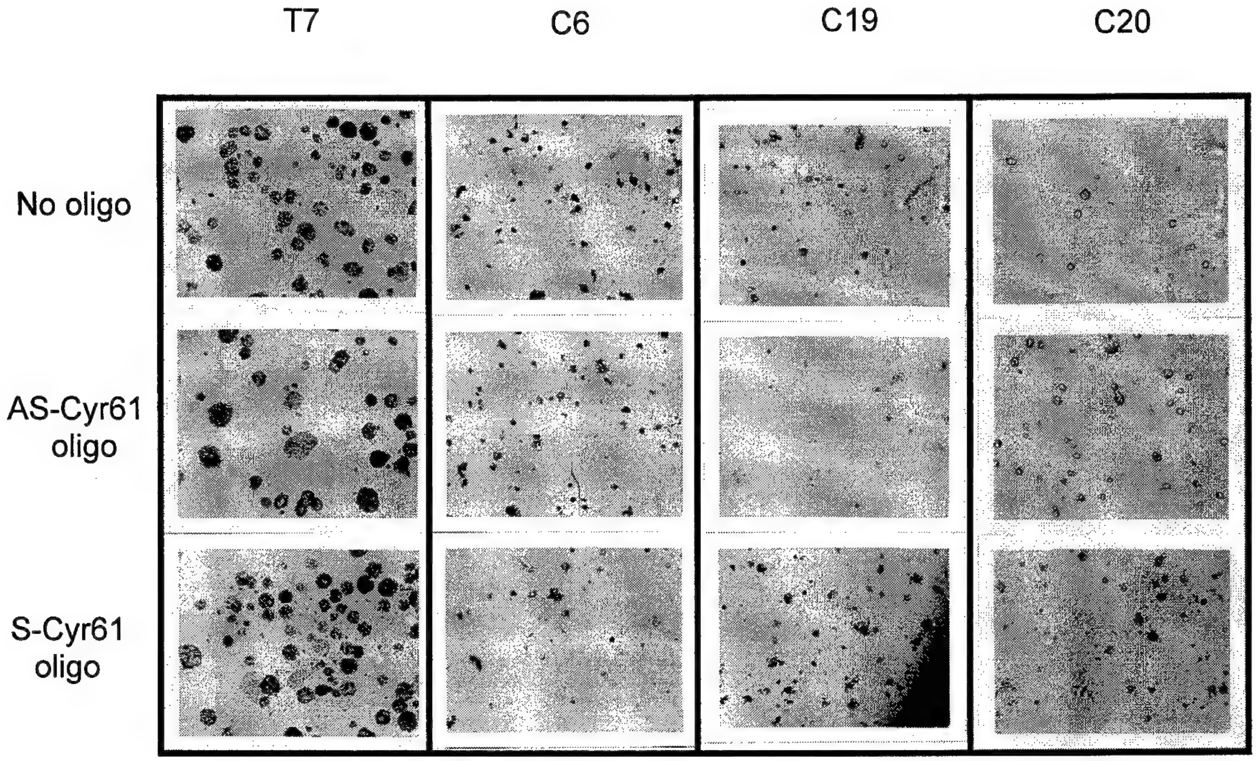

Figure 7. Blocking of Cyr61 expression by anti-sense Cyr61 oligonucleotides inhibits outgrowth of MCF-7 cells in Matrigel matrix. Subconfluent MCF-7/HRG clone T7 nd MCF-7/Cyr61 clones were cultured in E2-depleted media for 4 days and plated in triplicate in the presence or absence of control sense Cyr61 or anti-sense Cyr61 oligonucleotides. Outgrowth pattern was developed and photographed after 10-day incubation.

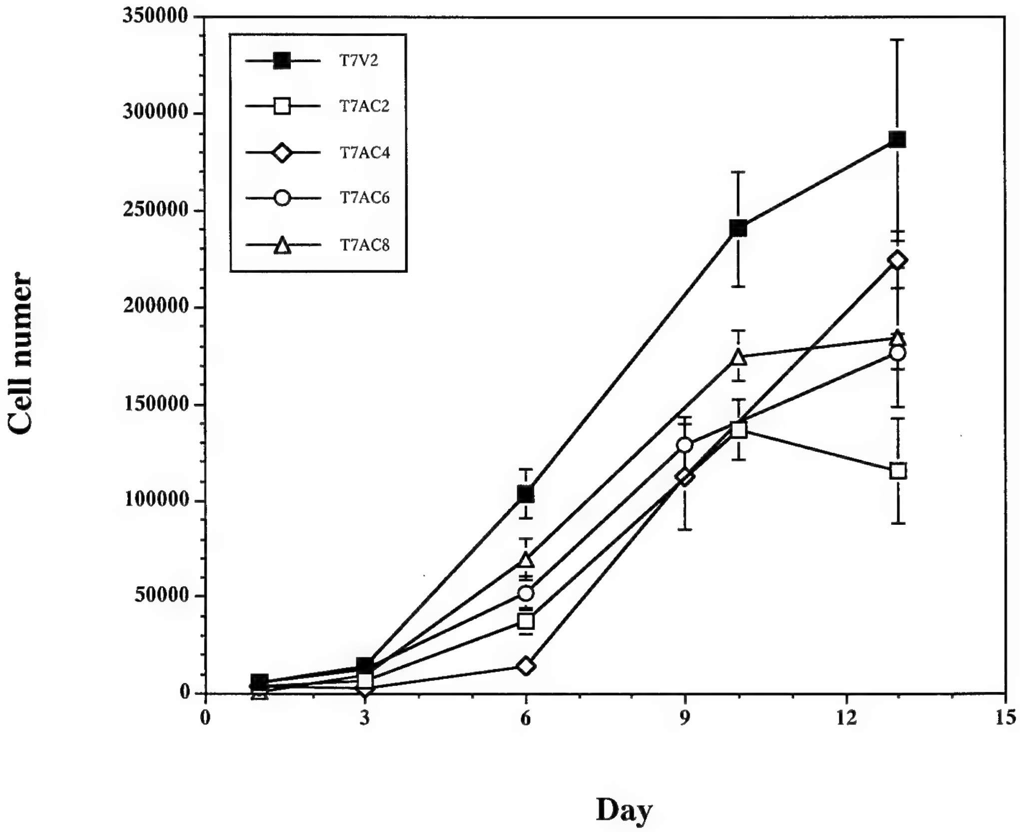

Figure 8. Proliferation of AS-Cyr61-transfected T7 cells is reduced as compared with parental T7 or vector control cells. HRG-ransfected MCF-7 cells (T7), vector control T7 cells (T7/V2) and AS-Cyr61-transfected-T7 clones (designated as T7AC2 and T7AC6) were plated (5,000 cells/well) in triplicate in 24-well plates. Cell number was determined at days 0, 3, 6, 9, and 13.

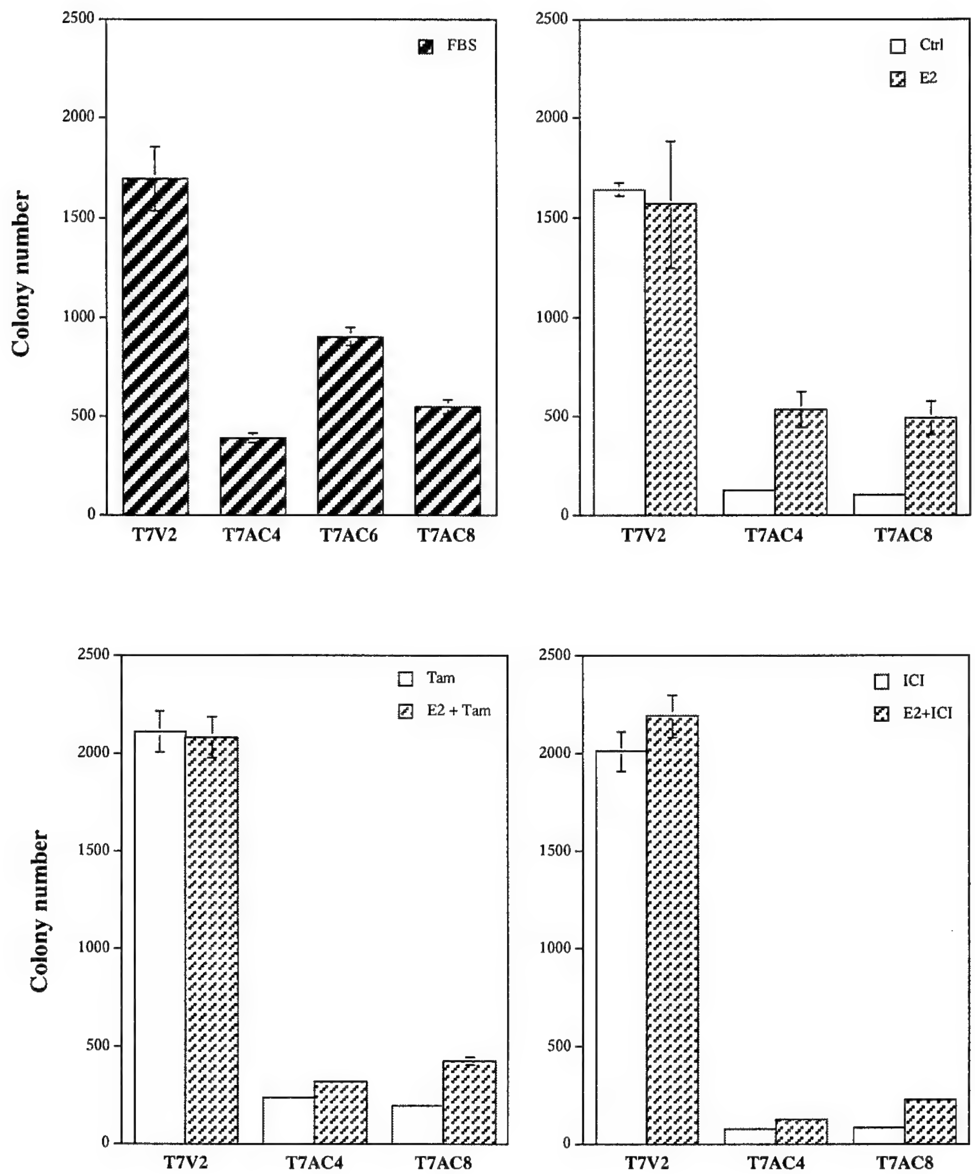

Figure 9. Antisense Cyr61-transfected T7 cells are E2-dependent and anti-E2-sensitive for both anchorage-dependent and anchorage-independent growth. (A) Cells were maintained in IMEM supplemented with 5% FBS and zeocin (200 μg/ml). When cells reached 80% confluency, the media was replaced by phenol red-free IMEM supplemented with charcoal-stripped FBS (CS-FBS) for four days to ensure complete depletion of estrogen. Cells were then plated (5,000 cells/well) in triplicate in 24-well plates and treated in the absence or presence of E2 (10^{-9} M), ICI (10^{-7} M) and the combination of both. Cell number was determined at days 0, 3, 6, 8, and 10. (B) Cells were maintained and treated as described above. Cells (10,000 cells)

were mixed with 0.35% agar and plated in duplicate onto a 0.6% agar layer in 6-well plates. Cells were treated with regular FBS media or with CS-FBS media in the absence or presence of E2 (10^{-9} M), Tam (10^{-7} M), ICI (10^{-7} M), and combinations of E2 and Tam, or E2 and ICI. Colony number was determined after 10-day culture.

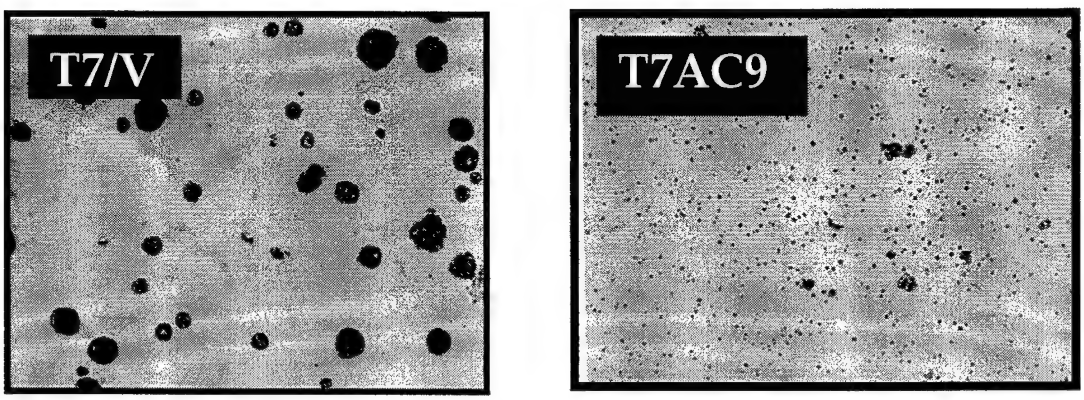

Figure 10. Anti-sense Cyr61 clones are incapable of invading and migrating through Matrigel matrix. MCF-7/HRG T7 (Vector or AS-Cyr61-transfected) were maintained in IMEM supplemented with 5% FBS. Cells (5,000 cells) mixed with Matrigel® were plated in duplicate in Matrigel coated 12-well plates. Media was added to the cells and microphotographs were taken after 10-day culture.

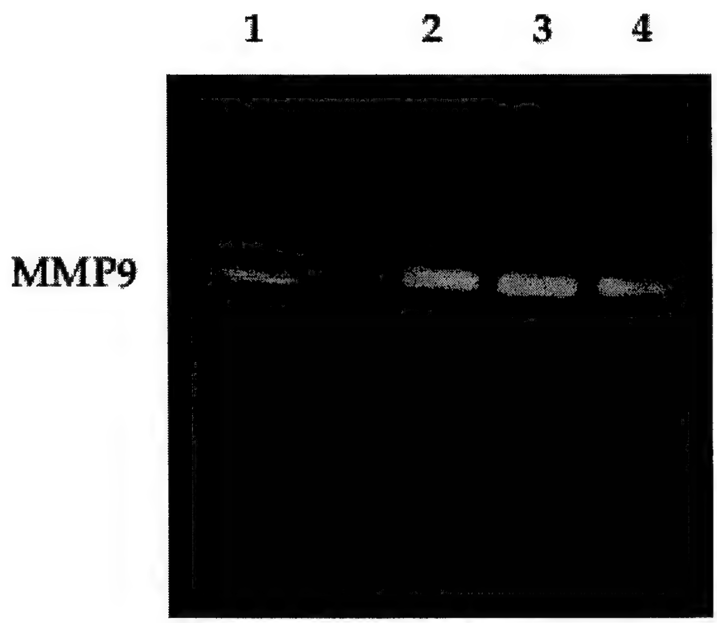

Figure 11. Matrix metalloproteinase 9 (MMP9) activity is decreased in anti-sense Cyr61-transfected cells. When cells in T150 flasks reached 80% confluency, the media was replaced with serum-free media for 24 hr followed by a second change of serum-free media for another 48 hr incubation. The conditioned media was collected and concentrated. Thirty micrograms of total protein was used for direct gelatin zymography analysis. Lane 1, the positive control; lane 2, vector cells; lane 3, T7AC3; and lane 4, T7AC9.

Table 1. Blockage of Cyr61 expression delays onset of tumor formation, and reduces size of tumors formed in athymic nude mice.

Cell line	Tumor size (mm3)	Cyr61 expression **
MCF-7*	NA	+/-
T7*	NA	+++++
T7/V2	354.8	++++++
T7AC2	241.3	+++++
T7AC4	16.6	+
T7AC6	20.2	_
T7AC8	13.2	-

NA, not available.

* Tumor sections obtained from Dr. Lupu's laboratory archives.

** Cyr61 expression was assessed by density of immunohistochemical (IHC) staining.

Key Research Accomplishments

- Expression of Cyr61 in MCF-7/Cyr61 clones was determined by Western blotting analysis.
- *In vitro* characterization of MCF-7/Cyr61 clones was completed, including matrigel outgrowth assays, gelatin zymography analysis, and steroid hormone response assays.
- The functional association of Cyr61 with MAPK, PI3K, and AKT signaling pathways was investigated.
- Initial *in vivo* characterization of MCF-7/Cyr61 clones was performed.
- Anti-sense Cyr61 oligonucleotides were tested on MCF-7/HRG and MCF-7/Cyr61 cells.
- Stable lines of MCF-7/HRG cells expressing anti-sense Cyr61 cDNA (designated as MCF-7/HRG/AS-Cyr61) were established.
- Initial *in vitro* characterization of MCF-7/HRG/AS-Cyr61 clones was performed, including anchorage-dependent growth assays, anchorage-independent soft agar colony formation assays, matrigel outgrowth assays, and gelatin zymography analysis.
- Initial *in vivo* characterization of MCF-7/HRG/AS-Cyr61 clones was performed, including anchorage-dependent growth assays, anchorage-independent colony formation assays in soft agar, Matrigel outgrowth assays, and MMP9 zymogram analyses.
- Determine the functional association of Cyr61 with MAPK, PI3K, and AKT signaling pathways.

Reportable Outcomes

Abstracts

2000 **Tsai, M.-S.** and Lupu, R. Cyr61, a ligand for integrin $\alpha v\beta 3$, modulates breast cancer progression. Abstract of the keystone symposia "Advances in Breast and Prostate Cancer", Incline Village, NA. (Poster presentation; abstract attached)

2000 **Tsai, M.-S.** and Lupu, R. Involvement of Cyr61, an angiogenic factor, in breast cancer tumor progression. The 1st International Workshop on the CCN Family of Genes, San Malo, France. (Abstract attached)

2000 **Tsai, M.-S.** and Lupu, R. Involvement of Cyr61, an angiogenic factor, in breast cancer tumor progression. Greece. (Abstract attached)

2001 **Tsai, M.-S.**, Mehmi, I., and Lupu, R. Cyr61 promotes breast cancer progression through regulation of the MAPK and AKT signaling pathways. Israel (Abstract attached)

Publications

2000 **Tsai, M.-S.**, Hornby, A.E, Lakins, J., and Lupu, R. Expression and function of Cyr61, an angiogenic factor, in breast cancer cell lines and breast tumor biopsies. Cancer Res. 60: 5603-5607. (Reprint attached)

2001 **Tsai, M.-S.**, Mehmi, I., Bogart, D. F., Castaneda, J. M., and Lupu, R. Cyr61 promotes breast cancer progression through regulation of the MAPK and AKT signaling pathways. (Submitted to Cancer Research; abstract attached)

2001 **Tsai, M.-S.,** Bogart, D. F., Li, P., Mehmi, I., and Lupu, R. Expression and regulation of Cyr61 in human breast cancer cell lines. (Submitted to Oncogene; abstract attached)

2001 **Tsai, M.-S.,** Bogart, D. F., Castaneda, J. M., Li, P. and Lupu, R. Blockage of Cyr61 expression reverses invasive and metastatic phenotypes of breast cancer cell lines. (Manuscript in preparation)

Awards

2000 Keystone Symposia Funded Scholarship

Conclusions

The second year training period of the DOD BCRP post-doctoral traineeship has allowed me to further master skills and techniques in biochemistry, cell biology, molecular biology, and animal physiology required essential for cancer research. It is evident in the research accomplishments described above. In addition, it has offered me with tremendous opportunities to establish extensive collaborations with breast cancer researchers. The provided travel funding has exposed me to better interactions with breast cancer advocates and survivors to guide our research direction timely to meet their needs. With the support from DOD, the preliminary data generated from the proposed project have enhanced our understanding at both the cellular and molecular levels of breast cancer progression, and should shed lights in developing Cyr61-targeted therapies to halt tumor progression and/or metastasis.

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Appendices

Title: Cyr61, a ligand for integrin $\alpha v\beta 3$, modulates breast cancer progression.

Authors: Tsai, M.-S. and Lupu, R.

Institute: Lawrence Berkeley National Laboratory, University of California, Berkeley, CA 94720.

Conference: Keystone Symposia meeting: Advances in Human Breast and Prostate Cancer (E1)

Location: Incline Village, NA, USA

Year: 2000

Abstract:

We have shown previously that heregulin (HRG), a growth factor that activates the erbB-2 receptor, is involved in breast cancer progression. We identified that Cyr61, a ligand for the integrin $\alpha v\beta 3$, is overexpressed in estrogen receptor-negative (ER^-), HRG^+, invasive, and metastatic breast cancer cells and tumors. To elucidate the role of Cyr61 in HRG induction of breast cancer progression, we blocked Cyr61 expression in HRG-overexpressing cells. Clones were isolated and characterized. We demonstrated that by blocking Cyr61 expression anchorage-dependent growth is significantly reduced and these cells lose their ability to grow in an anchorage-independent manner, as compared with the control cells. Moreover, blockage of Cyr61 expression promotes estrogen-independent cells to regain estrogen sensitivity, implying that Cyr61 plays a role in the acquisition of estrogen independence. Furthermore, abolishing Cyr61 expression promotes cells to lose their ability to invade and/or migrate through matrigel matrix. The mechanisms involved in these events apparently are mediated through regulation of the activity of extracellular matrix degrading proteins. Taken together, our results suggest that Cyr61 is a key regulator of breast tumor growth progression, i.e. anti-estrogen resistance, invasion and metastasis.

Title: Involvement of Cyr61, an angiogenic factor, in breast cancer tumor progression

Authors: Tsai, Miaw-Sheue and Lupu, Ruth

Institute: Lawrence Berkeley National Laboratory, University of California, Berkeley, CA 94720

Conference: The First International Workshop on the CCN Family of Genes

Location: San Malo, France

Year: 2000

Abstract:

Cyr61, a member of a newly identified CCN gene family, was isolated and identified by differential expression between estrogen receptor (ER)-positive and ER-negative breast cancer cells. Cyr61 is a ligand for the integrin $\alpha v\beta 3$. We showed that expression of Cyr61 in HRG-transfected MCF-7 cells is greatly increased compared to parental MCF-7 cells and it promotes invasion and metastasis. This phenotype is mediated, at least in part, by the regulation of MMP's and VEGF *in vivo*. We also showed that Cyr61 is expressed in all the invasive, metastatic, HRG-expressing, and ER-negative breast cancer cell lines. Moreover, Cyr61 was detected in about 30% of invasive human breast tumor biopsies. Most significantly, an anti-Cyr61 blocking antibody abolishes the invasiveness and migration of HRG-expressing breast cancer cells *in vitro*. Moreover, we have demonstrated that a function blocking anti-$\alpha v\beta 3$ antibody blocks Cyr61 induction of breast cancer cells invasion as measured by matrigel outgrowth. Recently, we demonstrated that Cyr61 is sufficient to confer estrogen independence and anti-estrogen resistance of breast cancer cells. In conclusion, these results strongly suggest that Cyr61 may play an important role in breast cancer progression possibly through regulation of invasion, metastasis, and neovascularization.

Title: Involvement of Cyr61, an angiogenic factor, in breast cancer tumor progression

Authors: Tsai, M.-S. and Lupu, R

Institute: Lawrence Berkeley National Laboratory, University of California, Berkeley, CA 94720

Conference:

Location: Olympia, Greece

Year: 2000

Abstract:

In searching for differentially expressed genes between estrogen receptor (ER)-positive (ER^+), non-metastatic and ER^-, metastatic breast cancer cells, the human homologue of a murine immediate-early gene, Cyr61, was isolated and identified. Cyr61 is a member of a newly identified CCN gene family and a ligand for the $\alpha v\beta 3$ integrin receptor. We demonstrated that expression of Cyr61 in HRG-transfected MCF-7 cells is greatly increased (5- to 20-fold) compared to parental MCF-7 cells, in which Cyr61 is nearly undetectable. Most recently, we have shown that Cyr61 is expressed in all the invasive, metastatic, HRG- expressing and ER^- breast cancer cell lines and highly expressed in about 30% of invasive human breast tumor biopsies tested. Most significantly, an anti-Cyr61 blocking antibody abolishes the invasiveness and migration of HRG-expressing breast cancer cell lines and HRG-transfected MCF-7 *in vitro.* We also showed that HRG-transfected MCF-7 expresses higher levels of the $\alpha v\beta 3$ integrin than parental MCF-7, indicating that these cells acquire angiogenic characteristics. Coincidently, the $\alpha v\beta 3$ integrin has been shown to be an angiogenic integrin and a prognostic indicator for breast carcinomas. Furthermore, we have recently demonstrated that Cyr61 is sufficient to confer estrogen independence and antiestrogen resistance of breast cancer cells. Taken together, these results strongly suggest that Cyr61 is a critical player in breast cancer progression through regulation of invasion, metastasis and neovascularization.

Title: Cyr61 promotes breast cancer progression through regulation of the MAPK and AKT signaling pathways.

Authors: Miaw-Sheue Tsai, Inderjit Mehmi, and Ruth Lupu.

Institute: Lawrence Berkeley National Laboratory, University of California, Berkeley, CA 94720

Conference:

Location: Israel

Year: 2001

Abstract:

We have previously identified a member of the CCN family of genes, Cyr61, as a gene potentially involved in breast cancer progression. Cyr61 is secreted into and predominantly incorporated into the extracellular matrix. We investigated whether expression of Cyr61 was necessary and/or sufficient to promote breast cancer cells to bypass their "normal" estrogen (E2) requirements. Aggressiveness of breast cancer cells is commonly attributed to the ability of these cells to overcome the E2 requirements for growth, and in most cases these carcinomas acquire anti-estrogen resistance. MCF-7 cells, which express high levels of estrogen receptor, are responsive to and dependent upon E2 for growth both *in vitro* and *in vivo*. Here we demonstrate that expression of Cyr61 in MCF-7 cells resulted in their progression from an E2-dependent to an E2-independent phenotype *in vitro* and *in vivo*. Our results clearly demonstrate that the MCF-7/Cyr61 cells show a growth advantage under serum-depleted conditions, which is consistent with the acquisition of an E2-independent phenotype. Significantly, these cells not only become E2-independent but also acquire an antiestrogen-resistant phenotype, which is one of the most common incident in breast cancer progression. The MCF-7/Cyr61 cells exhibited typical characteristics usually observed only in invasive breast carcinomas. The unique aggressiveness of the MCF-7/Cyr61 cells becomes evident by their Matrigel outgrowth pattern, their anchorage-independent growth, and their ability to form large and vascularized tumors in ovariectomized athymic nude mice. We also demonstrate that the mechanism by which Cyr61 achieves the induction of tumorigenicity in breast cancer cells is mediated via the specific activation of the MAPK and AKT signaling pathways, and that Cyr61 expression regulates the activity of a matrix-degrading enzyme, MMP-9. Taken together, our results suggest that Cyr61 is

a key regulator of breast cancer progression, mediated at least in part via the activation of the MAPK and AKT signaling pathways as well as activation of a matrix-degrading enzyme. Therefore, our current studies are aimed at developing strategies to block Cyr61 expression and its binding to its receptors.

[CANCER RESEARCH 60, 5603–5607, October 15, 2000]

Advances in Brief

Expression and Function of CYR61, an Angiogenic Factor, in Breast Cancer Cell Lines and Tumor Biopsies[1]

Miaw-Sheue Tsai, Ann E. Hornby,[2] Johnathon Lakins,[3] and Ruth Lupu[4]

Ernest Lawrence Berkeley National Laboratory, University of California, Berkeley, California 94720

Abstract

We have previously shown that expression of heregulin (HRG) is closely correlated with breast cancer progression. We have subsequently isolated Cyr61, a ligand for the $\alpha_v\beta_3$ integrin that is differentially expressed in HRG-positive cells, and have shown that it is expressed in all of the invasive and metastatic breast cancer cell lines tested. Preliminary evaluation of Cyr61 expression in breast tumor biopsies revealed expression of Cyr61 in about 30% of invasive breast carcinomas. Significantly, we demonstrated that Cyr61 is a downstream effector of HRG action, because a Cyr61-neutralizing antibody abolished the ability of HRG-expressing cells to migrate *in vitro*. Furthermore, we have shown that HRG-expressing cells denote higher levels of $\alpha_v\beta_3$ expression, and we have established that Cyr61 action is mediated, at least in part, through its receptor $\alpha_v\beta_3$, because a functional blocking antibody of the $\alpha_v\beta_3$ blocked the Matrigel outgrowth of HRG-expressing cells. These results strongly suggest that Cyr61 is necessary for HRG-mediated chemomigration and that Cyr61 plays a functional role in breast cancer progression, possibly through its interactions with the $\alpha_v\beta_3$ receptor.

Introduction

Many E2[5]-dependent and antiestrogen-responsive breast tumors spontaneously progress to an E2-independent and antiestrogen-resistant phenotype, becoming deadly metastatic diseases. The mechanism by which breast cancer appears to progress from an E2-dependent to an E2-independent phenotype is still under investigation. We have shown that expression of HRG, an activator of *erb*B-2/-3/-4 receptor signaling pathways, is closely associated with an invasive breast cancer phenotype (1). Furthermore, we demonstrated that HRG induces breast cancer progression, as determined by loss of ER function and response, tumorigenicity (2), invasion (3), and metastasis (4). It has been hypothesized that HRG induces activation of the *erb*B signaling pathways, leading to regulation of downstream genes that regulate and control cancer progression. Therefore, to develop effective targeted therapies, it is important to identify gene(s) directly involved in HRG-induced breast cancer aggressiveness.

With this in mind, we have isolated and identified the human homologue of a mouse immediate-early response gene, *Cyr61*, differentially expressed in ER-negative, HRG-positive breast cancer cells. Cyr61 is a secreted cysteine-rich protein that is associated with the cell surface and the extracellular matrix (5). Cyr61 mediates cell adhesion, migration, and angiogenesis (6, 7). In this report, we establish that Cyr61 is coexpressed with HRG in all of the metastatic breast cancer cell lines tested, its expression is inversely correlated with ER expression, and it is associated with HRG-induced breast cancer chemomigration and metastasis, possibly through interactions with the $\alpha_v\beta_3$ integrin receptor. Furthermore, we establish that Cyr61 was expressed in about 30% of invasive breast cancer tumor biopsies, implying a possible role in breast cancer progression.

Materials and Methods

Cells and Cell Culture

Breast cancer cell lines were obtained from the American Type Culture Collection and routinely cultured in phenol red-containing improved MEM supplemented with 5% (v/v) fetal bovine serum and 2 mM L-glutamine at 37°C in a humidified atmosphere with 5% CO_2, unless otherwise specified.

Plasmids and Generation of Riboprobes

A Cyr61 riboprobe plasmid was constructed by cloning a PCR fragment of Cyr61 cDNA into the pCRII TA cloning vector (Invitrogen). The sequence of primers used to generate the Cyr61 fragments was as follows: (*a*) forward primer, 5′-TGTGGAACTGGTATCTCCACACGA-3′ (nucleotides 727–750); and (*b*) reverse primer, 5′-TCTTTTCACTGAATATAAAATTAAAA-3′ (nucleotides 1739–1764). The Cyr61 riboprobe construct was sequenced using Sequenase v.2.0 with ^{35}S-labeled dCTP. Radioactive riboprobe was prepared by linearizing the plasmid with the restriction enzyme *Dde*I, which generated a 524-bp fragment, and followed by reverse transcription *in vitro* using the SP6 RNA polymerase in the presence of [^{32}P]UTP. The riboprobe plasmid of GAPDH was kindly provided by Dr. Francis Kern (University of Alabama, Birmingham, AL). Radiolabeled GAPDH riboprobe was generated using T7 RNA polymerase as described above, except that it was linearized by the restriction enzyme *Bam*HI.

RNase Protection Assay

Total RNA was extracted by Tripure isolation solution (Roche Molecular Biochemicals) and quantified by spectrophotometry. RNA (30 μg) was hybridized with 100,000 cpm of ^{32}P-labeled Cyr61 riboprobe for 12–16 h at 50°C. ^{32}P-labeled GAPDH riboprobe (10,000 cpm) was added to each sample as an internal control. Hybridized RNA samples were digested with 25 μg of RNase A for 30 min at 28°C. The reaction was terminated by incubating with proteinase K (250 μg/ml) and 0.5% SDS for 15 min at 37°C. After phenol extraction, RNA samples were coprecipitated with 10 μg of yeast tRNA in absolute ethanol. RNA was redissolved in a denaturing loading buffer and resolved by electrophoresis on a 6% polyacrylamide-urea gel. Protected fragments of Cyr61 (305 bp) and GAPDH (100 bp) were visualized by autoradiography. [^{32}P]dCTP-end-labeled pBR322/*Msp*I (New England Biolabs) was used as a molecular weight marker.

Western Blot Analysis

Cyr61 Present in Conditioned Media. Subconfluent human breast cancer cells were maintained in serum-free media for 3–4 days. The conditioned media were collected, and the Cyr61 protein was purified by heparin affinity

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[5] The abbreviations used are: E2, estradiol; Tam, tamoxifen; ER, estrogen receptor; HRG, heregulin; GAPDH, glyceraldehyde-3-phospate dehydrogenase.

chromatography. The column was washed with 0.3–0.6 M NaCl in 10 mM Tris-HCl (pH 7.5). The Cyr61 protein was eluted at 0.9 M NaCl and desalted by PD-10 Sephadex G25M columns (Amersham-Pharmacia). The eluted fractions were concentrated (10×) and resolved by 12% Tris-glycine SDS-PAGE. The separated proteins were electroblotted onto a Hybond enhanced chemiluminescence nitrocellulose membrane (Amersham-Pharmacia). The blotted membrane was blocked overnight at 4°C with 5% (w/v) BSA in Tris-buffered saline containing 0.5% Tween 20 (TBST) and incubated with the rabbit anti-Cyr61 polyclonal antibody (1:5,000 dilution) for 1 h at room temperature. After three washes with TBST, the blot was incubated with a 1:10,000 dilution of horseradish peroxidase-linked donkey antirabbit IgG secondary antibody. The Cyr61 protein was detected by the enhanced chemiluminescence reaction using Hyperfilm (Amersham-Pharmacia).

Cyr61 Present in Breast Cancer Tumor Specimens. Breast tumor specimens were lysed in radioimmunoprecipitation assay buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5 mM EDTA, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, and 1 mM DTT] with protease inhibitors (10 μg/ml leupeptin, 10 μg/ml aprotinin, 0.5 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride). Protein concentration was determined by a Micro BCA detection reagent kit (Pierce). Equal amounts of proteins were loaded and separated by SDS-PAGE followed by Western blot analysis as described above.

Immunohistochemical Staining

Formalin-fixed paraffin-embedded breast tumor sections were deparaffinized in xylene and hydrated in a graded alcohol series. Slides were quenched for endogenous peroxidase activity in the presence of 0.3% H_2O_2 for 30 min and blocked with 10% (v/v) horse serum for 30 min. Slides were then incubated with a polyclonal anti-Cyr61 antibody (1:5000) overnight at 4°C. The sections were washed in PBS before the incubation with a biotinylated antirabbit IgG secondary (1:200) antibody for 30 min. The sections were then incubated with an avidin-biotin complex (VECTASTAIN Elite ABC reagent; Vector Laboratories) for 30 min, and the reaction was developed in the presence of hydrogen peroxide and 3,3′-diaminobenzidine tetrahydrochloride. The slides were counterstained with hematoxylin solution and mounted with the aqueous Crystal mount media.

Chemomigration and Chemotaxis Assays

Boyden chamber chemomigration assays were performed using a 48-well chemotaxis chamber (Neuro Probe). Breast cancer cells (20,000 cells/well) were plated onto the upper chambers in triplicate or quadruplicate onto a 12 μm polycarbonate filter membrane coated with collagen (Becton Dickinson). The conditioned media derived from NIH3T3 fibroblast culture was used as a chemoattractant in the lower chambers. Cells were incubated at 37°C in a humidified 5% CO_2 atmosphere for 16 h. After the incubation, the membrane was removed from the chamber. The cells on the top surface were removed, and the cells on the bottom side of the membrane were fixed in methanol and stained with a Diff-Quick Stain kit. Membranes were then mounted onto glass slides, and the cells that migrated through the pores to the opposite side of the membrane (bottom side) were quantified using a light microscope.

Matrigel Outgrowth Assay

Cells (5,000 cells/well) were mixed with 150 μl of Matrigel (Becton Dickinson) and plated in triplicate onto the Matrigel-coated 12-well plates for 1 h at 37°C. Cells were then cultured in the media containing the indicated concentrations of antibodies for 7–10 days. The pattern of the cells' outgrowth in Matrigel matrix was examined and photographed using a phase-contrast microscope.

Results and Discussion

Cyr61 Is Differentially Expressed in HRG-positive *versus* HRG-negative Breast Cancer Cell Lines. We have demonstrated that expression of HRG is highly associated with aggressive progression of breast cancers to hormone independence, antiestrogen resistance, invasion, and metastasis (2–4). To identify genes that were involved in the HRG induction of breast cancer progression, a number of genes were isolated and cloned by differential expression in MDA-MB-231 HRG-expressing cells. Sequence and homology analyses indicated that one of the genes is the human homologue of a mouse immediate-early response gene, *Cyr61*. Cyr61 was highly and selectively expressed in MCF-7/HRG [MCF-7/HRG (HRG-transfected MCF-7) clones, *e.g.*, T2, T6, T7, and T8] but was nearly undetected in MCF-7/V cells (vector-transfected MCF-7 cells). A 5–25-fold increase in the Cyr61 mRNA level was observed in MCF-7/HRG cells as compared with MCF-7/V cells. HRG-positive MDA-MB-231 cells also expressed high levels of Cyr61 (Fig. 1*A*). To determine whether the protein was also selectively expressed, we performed Western blot analysis and immunohistochemistry using an anti-Cyr61 polyclonal antibody. As shown in Fig. 1*B*, Cyr61 protein expression was observed in the MCF-7/HRG cells but not in the vector control cells. These studies were performed using cells cultured under serum-depleted conditions.

In addition, immunohistochemical staining was performed on paraffin sections of MCF-7/HRG and MCF-7/V tumors formed in xenografted athymic nude mice. These tumors were observed as a mixture of solid, trabecular, and tubular patterns. Irregular gland formation and occasional well-formed lumen were present. These features, as well as the heterogeneity and variety of histological patterns, resemble those observed in mammary infiltrating ductal carcinoma known as no special type (Fig. 1*A*). Similar features were observed in all of the tumors examined. As can be seen in Fig. 1*C*, Cyr61 expression was very predominant in MCF-7/HRG-derived tumors (*right panel*). Expression of the Cyr61 protein was localized to the cytoplasm of the tumor cells, whereas only a weak staining of Cyr61 was observed in tumors derived from MCF-7/V cells supplemented with E2 (*left panel*). Once again, our data demonstrate a differential expression of Cyr61 in HRG-expressing cells.

We mapped the human *Cyr61* gene to chromosome 1p (data not shown), consistent with previous studies showing the localization of Cyr61 to chromosome 1p22.3 (8, 9). Abnormalities of chromosome 1p have correlated with ER negativity and a poor prognosis in breast cancer (10) and other malignancies (11–13).

It has been shown that murine Cyr61 is regulated by 12-*O*-tetradecanoylphorbol-13-acetate in the liver (14), as well as by E2 and Tam in the uterus (15). We have shown that the human homologue of Cyr61 is regulated by E2 and several antiestrogens including Tam and ICI 182,780 in ER-positive breast cancer cells.[6] The induction of Cyr61 was most significant in MCF-7 cells [up to a 10–12-fold increase by 6 h of treatment with E2 (10^{-9} M) or 3 h of treatment with ICI 182,780 (10^{-7} M)] and to about a 5–6-fold increase with Tam (10^{-7} M). On the other hand, the up-regulation of Cyr61 was not significant in HRG-expressing cells, with an increase of only 1.5–2-fold by any of the treatments (data not shown). These results are consistent with our published data demonstrating that HRG promotes an estrogen-independent phenotype and that HRG blocks ER function resulting in MCF-7/HRG cells that fail to respond to E2 and the consequential inability of E2 to induce the expression of E2-regulated genes.

Cyr61 Is Overexpressed in HRG-positive, ER-negative Breast Cancer Cell Lines. To assess whether up-regulation of Cyr61 expression was a result of HRG overexpression in MCF-7/HRG cells or whether it was a common theme occurring in breast cancer cells, we examined its expression in many human breast cancer cell lines. Basal level of Cyr61 expression was measured in cells cultured under serum-depleted conditions to prevent the influence of E2 on Cyr61

[6] M. S. Tsai, E. Gilad, M. Cardillo, and R. Lupu. Heregulin (HRG) promotes tumor formation, manuscript in preparation.

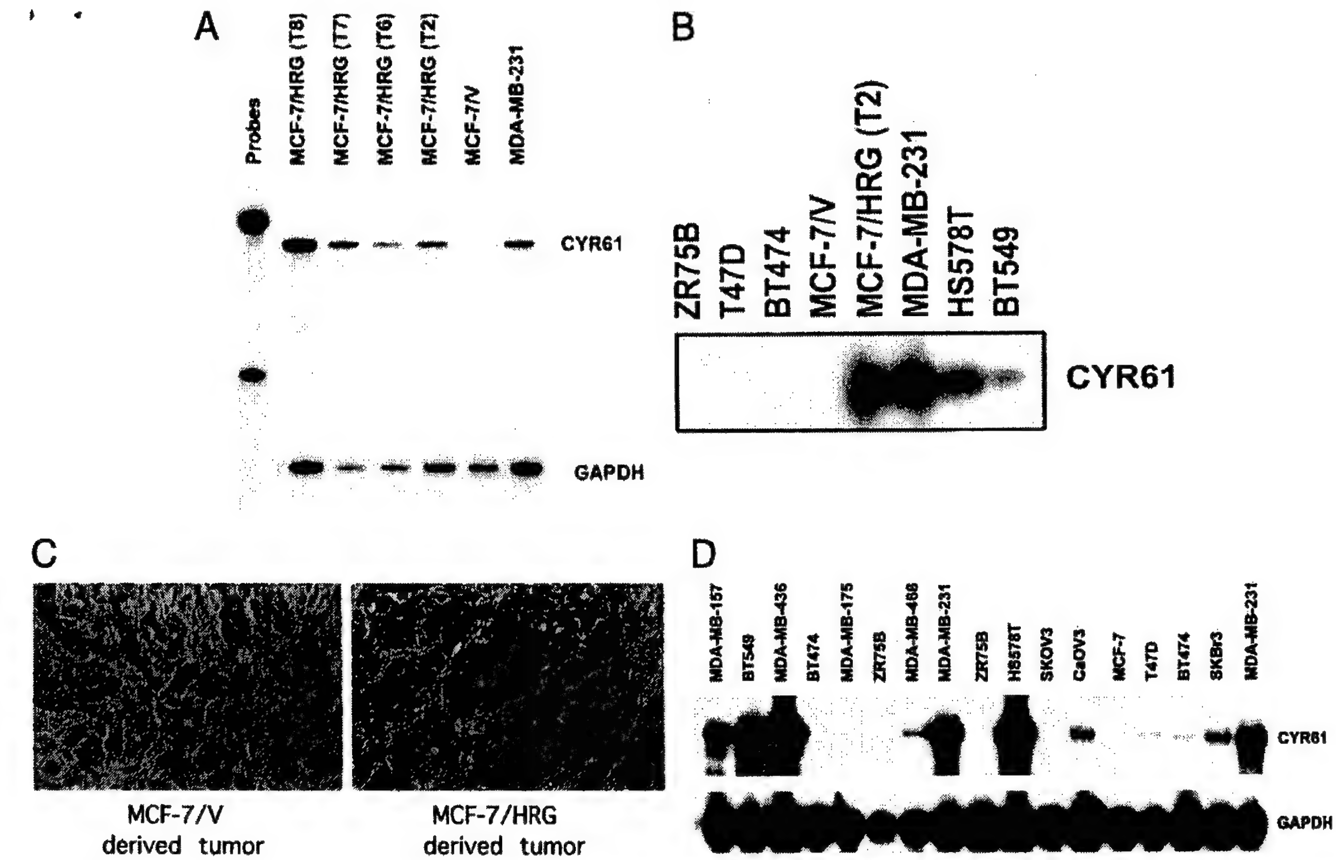

Fig. 1. Expression of Cyr61 in breast cancer cells. *A*, subconfluent MCF-7/HRG and MCF-7/V cells were maintained in serum-depleted conditions for 3 days. Total RNA was isolated, and 30 μg of RNA were analyzed by RNase protection assay. RNA from MDA-MB-231 cells was used as a positive control for Cyr61 expression. The GAPDH probe was used as an internal control for RNA loading. *B*, subconfluent breast cancer cell lines were cultured in serum-free media. Conditioned media were collected after 3 days, and heparin column chromatography was performed. The Cyr61 protein was eluted and analyzed by Western blotting analysis as described in "Materials and Methods." *C*, immunohistochemical analysis was performed as described in "Materials and Methods" for MCF-7/V- and MCF-7/HRG-derived tumor sections. *D*, breast cancer cells were cultured as described in *A*, and total RNA was isolated and analyzed by RNase protection assay.

expression. As shown in Fig. 1, *B* and *D*, a tight correlation between Cyr61 mRNA and protein expression exists in all of the cell lines tested. Cyr61 is highly expressed in MDA-MB-231, HS578T, BT549, and MCF-7/HRG cells, all of which are HRG-expressing and ER-negative cells, but it is low or undetectable in cells that do not express HRG and are ER-positive, including MCF-7, ZR75B, T47D, and BT474 cells. These studies were performed by RNase protection assays and by Western blot analysis in which a secreted M_r 45,000 protein derived from conditioned media was detected using an anti-Cyr61 polyclonal antibody.

Our data indicate that a high level of Cyr61 expression correlates with HRG expression and inversely correlates with ER expression, response to E2, and sensitivity to antiestrogens (16). Moreover, the expression of Cyr61 strongly correlates with vimentin expression, a known marker for invasiveness (17), and is associated with the ability of breast cancer cells to invade *in vitro* and metastasize *in vivo*. On the other hand, low to undetectable levels of Cyr61 expression were seen only in the HRG-negative, ER-positive, E2-dependent, antiestrogen-sensitive breast cancer cells. These data are summarized in Table 1. Taken together, these data show that Cyr61 expression is associated with HRG expression and is apparently linked to breast cancer progression. Because *Cyr61* is an early response gene, it could be argued that its expression would be up-regulated in rapidly proliferating cells. Thus, it is critical to establish that up-regulation of Cyr61 in MCF-7/HRG cells is not attributable to a proliferative advantage of these cells. Cell cycle analysis by flow cytometry demonstrated that no differences in cell cycle distribution were observed between the MCF-7/HRG cells and the parental MCF-7 cells (18).

Cyr61 Is Expressed in about 30% of Breast Tumor Biopsies. To determine whether expression of Cyr61 may have clinical relevance in breast cancer, its expression in biopsies was determined. A pilot study was performed using Western blot analysis on proteins extracted from paraffin sections. Forty percent (4 of 10) of the tumor specimens, all of which were ER-negative invasive breast carcinomas, showed high expression of the Cyr61 protein (Fig. 2*A*). Total cell lysates of MDA-MB-231 and MCF-7 were used as positive and negative controls, respectively. It is noteworthy that Cyr61 protein expression was low in cell lysates of MDA-MB-231, because Cyr61 is mostly secreted to the cultured media. Additional studies revealed that Cyr61 was detected in about 30% of breast tumor specimens (n = 55) by immunohistochemistry (Fig. 2*B*). Cyr61 staining was demonstrated to be specific because it was completely blocked in the presence of excess recombinant Cyr61 protein (data not shown). No staining was observed in normal components of the biopsies. These data suggest that in at least 30% of these tumors, Cyr61 may be required for survival; therefore, it may be strongly implicated in breast cancer progression.

An Anti-Cyr61-Neutralizing Antibody Blocks Chemomigration of MCF-7/HRG Cells. To demonstrate that Cyr61 is a direct downstream regulator of HRG action, studies were performed using a Cyr61-neutralizing antibody [Refs. 5–7; kindly provided by Dr. Lester F. Lau (University of Illinois, Chicago, IL)]. For these studies, we used MCF-7/HRG cells, which have been shown to migrate through collagen in a Boyden chamber assay (as shown below). Cells were treated with increasing concentrations of the antibody, and the ability of the cells to migrate *in vitro* was assessed. The anti-Cyr61-neutralizing antibody inhibited migration of MCF-7/HRG cells in a dose-

Table 1 *Expression of Cyr61 in breast cancer cell lines*[a]

Cell line	Cyr61	HRG	ER	Invasive *in vitro*	Metastatic *in vivo*	$\alpha_v\beta_3$[b]
MCF-7	−	−	++++	−[c]	−	+/−
T47D	−	−	++	−[c]	−	−
BT474	−	−	++	−[c]	−	−
MDA-MB-175	−	+/−[d]	+	−[c]	−	ND
ZR75B	−	+/−[d]	+	−[c]	−	−
MDA-MB-468	+	−	−	+[e]	−	−
SKBR-3	+	−	−	+[e]	−	−
MDA-MB-157	++	++	−	+	ND	ND
MDA-MB-436	+++	+++	−	+++	ND	ND
BT-549	+++	+++	−	+++	+	ND
MDA-MB-231	++++	++++	−	++++	+	+++
MDA-MB-435	++++	++++	−	++++	+	+++
HS578T	++++	++++	−	++++	+	ND
MCF-7/HRG	+++	+++	−/+	+++	+	++

[a] − indicates no expression; the number of plus signs indicates the increase in expression.
[b] The $\alpha_v\beta_3$ integrin expression is based on results from Ref. 19 and our preliminary data. ND, not determined.
[c] Cells require E2 for invasion *in vitro* and growth *in vivo* and never metastasize *in vivo*.
[d] E2 induces expression of HRG.
[e] Cells require ligand (epidermal growth factor or HRG) to invade but never metastasize *in vivo*.

dependent manner (Fig. 3*A*). No effect was observed when a control IgG antibody was used under the same conditions and concentrations. Similar results were observed in other invasive, HRG-expressing breast cancer cells, such as MDA-MB-231, HS578T, and BT549 (data not shown). These studies suggest, for the first time, a possible association between the increase in Cyr61 expression and the invasive potential triggered by HRG. Additional studies are required to assess the direct association between Cyr61 and HRG and their joint action resulting in breast cancer progression. Of note, the anti-Cyr61-neutralizing antibody had no effect on MCF-7 cells. It is important to note that MCF-7 cells do not migrate through collagen.

The $\alpha_v\beta_3$ Integrin Receptor Is Involved in Cyr61-Mediated Breast Cancer Progression. Because Cyr61 was shown to be a ligand for the $\alpha_v\beta_3$ integrin (19), we speculated whether Cyr61 requires expression of $\alpha_v\beta_3$ for its action. Thus, we assessed the level of $\alpha_v\beta_3$ expression in MCF-7/HRG cells and showed that the level of $\alpha_v\beta_3$ was augmented in MCF-7/HRG cells compared with the MCF-7/V cells (data not shown), as determined by immunofluorescence staining using an anti-$\alpha_v\beta_3$ antibody on cultured cells. We then speculated that if the action of Cyr61 is mediated through the $\alpha_v\beta_3$ receptor, it is plausible that blockage of the $\alpha_v\beta_3$ integrin will modulate the growth characteristics of MCF-7/HRG cells. Thus, Matrigel outgrowth and migration studies were performed in the presence and absence of an anti-$\alpha_v\beta_3$ functional blocking antibody. We determined that this antibody specifically blocked the Matrigel outgrowth of HRG-expressing cells in a dose-dependent manner (Fig. 3*B*). No effects were observed when control IgG was used. Similar inhibitory effects of the anti-$\alpha_v\beta_3$ antibody were seen in HRG-positive MDA-MB-231 cells (data not shown).

The results indicated that the functional $\alpha_v\beta_3$ integrin is required for maintaining the invasive capacity of HRG-expressing cells, and that the aggressive phenotypes induced by HRG are mediated, in part if not entirely, by Cyr61 and its receptor, $\alpha_v\beta_3$ integrin. Because Cyr61, an angiogenic factor, and its receptor, $\alpha_v\beta_3$, are both induced

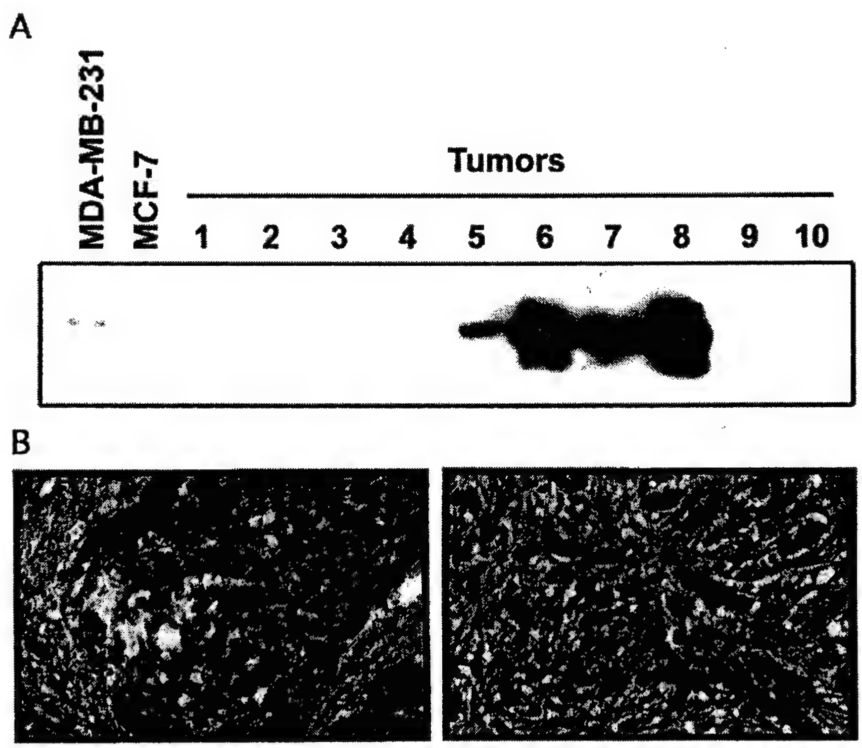

Fig. 2. Expression of Cyr61 in human breast tumor biopsies. *A*, human breast tumors were lysed in radioimmunoprecipitation assay buffer, and equal amounts of protein were resolved on a 4–20% gradient SDS-polyacrylamide gel. Western blotting analysis was performed as described in "Materials and Methods." Cell lysates of MCF-7 and MDA-MB-231 cells were used as negative and positive controls, respectively. *B*, immunohistochemical analysis of human breast carcinoma biopsies was performed as described in "Materials and Methods." Two representative microphotographs are shown for Cyr61 expression.

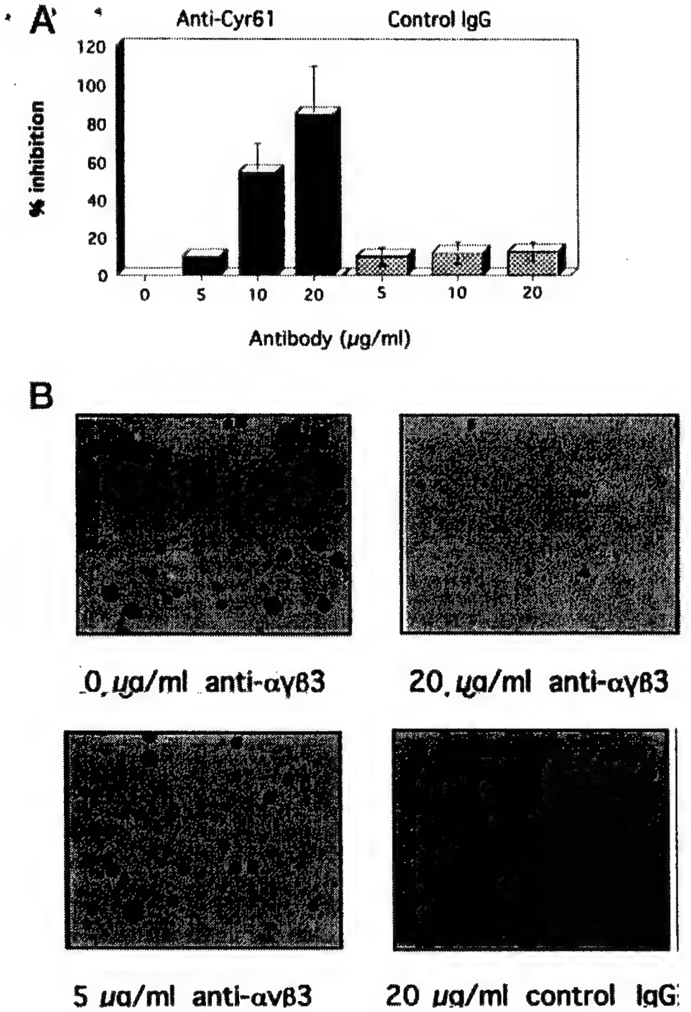

Fig. 3. *A*, blocking the invasive phenotypes of HRG-expressing cells by a Cyr61-neutralizing antibody. MCF-7/HRG cells were treated in the absence or presence of increasing concentrations (5, 10, and 20 μg/ml) of the anti-Cyr61 antibody or a control IgG for 16 h in the Boyden chamber assay. Chemomigration was measured based on the number of cells traversing collagen-coated filters. Data are the mean of triplicates from a representative experiment. SD was calculated for each data point. *B*, inhibited outgrowth of MCF-7/HRG cells by a functional blocking antibody of $\alpha_v\beta_3$. MCF-7/HRG cells were treated in the absence or presence of increasing concentrations of the anti-$\alpha_v\beta_3$ antibody (LM609; only the 5 and 20 μg/ml concentrations of antibody are shown) or a control IgG (20 μg/ml) in Matrigel outgrowth assay for 7 days. Outgrowth pattern was examined and photographed.

in the MCF-7/HRG cells, it is tempting to postulate that these factors are involved in the increased neovascularization that we have observed in the tumors formed by MCF-7/HRG cells in athymic nude mice.[6] The exact mechanism by which HRG promotes an aggressive breast cancer phenotype is still unknown. However, the identification of Cyr61 expression in breast cancer tumor progression is of great significance, especially because its receptor, the $\alpha_v\beta_3$ integrin, was recently shown to be a good prognostic indicator in breast cancer (20, 21). Studies are under way to determine whether ectopic expression of Cyr61 alone, in HRG-negative cells, is sufficient and/or necessary to confer some biological activities induced by HRG, such as loss of E2 response, acquisition of antiestrogen resistance, and chemomigration.

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Title: Cyr61 promotes breast cancer progression through regulation of the MAPK and AKT signaling pathways[1]

Authors: Miaw-Sheue Tsai, Inderjit Mehmi, Daphne F. Bogart, Jessica M. Castañeda, and Ruth Lupu

Submitted: Cancer Research, 2001

Abstract:

We have previously identified a member of the CCN family of genes, Cyr61, as a gene potentially involved in breast cancer progression. Cyr61 is secreted and predominantly incorporated into the extracellular matrix. Recently, we have shown that Cyr61 was overexpressed in invasive and metastatic human breast cancer cells and tissues. In addition, we demonstrated that Cyr61 function is necessary for heregulin (HRG)-mediated chemomigration of breast cancer cells. In the present study, we investigated whether expression of Cyr61 is necessary and/or sufficient to promote breast cancer cells to bypass their "normal" estrogen (E2) requirements. Aggressiveness of breast cancer cells is commonly attributed to the ability of these cells to overcome the E2 requirements to growth, and in most cases these carcinomas acquire anti-estrogen resistance. MCF-7 cells, which express high levels of estrogen receptor, are responsive to and dependent upon E2 for growth both *in vitro* and *in vivo*. Here we demonstrate that, while expression of Cyr61 in MCF-7 cells resulted in their progression from an E2-dependent to an E2-independent phenotype *in vitro* and *in vivo*, these cells are still responsive to E2 to the same extent as the parental cells. Our results clearly demonstrate that the MCF-7/Cyr61 cells show a growth advantage under serum-depleted conditions, which is consistent with the acquisition of an E2-independent phenotype. Significantly, these cells not only become E2-independent but also acquire an antiestrogen-resistant phenotype, which is one of the most common incidents in breast cancer progression. All of the MCF-7/Cyr61 clones (more than 5 clones) exhibited typical characteristics usually observed only in invasive breast carcinomas but seldom in the control MCF-7/V cells. The unique aggressiveness of the MCF-7/Cyr61 cells becomes evident by their Matrigel outgrowth pattern, which is irregular with markedly larger colonies, and these cells become anchorage-independent. Moreover, these cells are tumorigenic in athymic nude mice when inoculated into the mammary fat pad, and the tumors are highly vascularized. Recently, we demonstrated that blockage of $\alpha v\beta 3$, one of the integrin receptors to

which Cyr61 binds, blocks Cyr61 induction of breast cancer progression. However, the mechanism by which Cyr61 promotes this progression remains unknown. Here, we demonstrate that a mechanism by which Cyr61 achieves the induction of tumorigenicity in breast cancer cells is mediated via the specific activation of the MAPK and AKT signaling, and that Cyr61 expression regulates the activity of a matrix-degrading enzyme, MMP-9, also known as Gelatinase B. Taken together, our results suggest that Cyr61 is a key regulator of breast cancer progression. Cyr61 is involved in the ability of the cells to bypass their normal estrogenic requirements, at least in part, via the activation of the MAPK and AKT signaling pathways, and to promote invasion and metastasis possibly via the regulation of an enzyme that is capable of degrading the extracellular matrix (MMP-9). To our knowledge, this is the first report indicating a specific mechanism by which Cyr61 promotes breast cancer tumor growth *in vivo*.

Title: Expression and regulation of Cyr61 in human breast cancer cell lines

Authors: Miaw-Sheue Tsai, Daphne F. Bogart, Patricia Li, Inderjit Mehmi, and Ruth Lupu.

Submitted: Oncogene

Abstract:

We have shown that Cyr61, an angiogenic regulator, is overexpressed in invasive and metastatic human breast cancer cells and tumor biopsies. We have further demonstrated that Cyr61 promotes acquisition of estrogen-independence and anti-estrogen resistance *in vitro* in breast cancer cells. Moreover, we have demonstrated that Cyr61 induces tumor formation and tumor vascularization *in vivo*, events mediated through the activation of the MAPK and the Akt signaling pathways. Here we investigate how Cyr61 expression is regulated in both estrogen receptor (ER)-positive and ER-negative breast cancer cells. We demonstrate that Cyr61 mRNA and protein expression is inducible by estrogen and anti-estrogens in ER-positive breast cancer cells. We show that a labile protein as well as a negative regulator might be involved in Cyr61 expression in estrogen-dependent breast cancer cells. Other important regulators of Cyr61 expression in breast cancer cells that we found are the phorbol ester TPA, vitamin D, and retinoic acid. TPA causes positive regulation of Cyr61 expression in ER-positive MCF-7 cells. Vitamin D induces a transient stimulatory effect on Cyr61 gene expression. Lastly, retinoic acid has a negative effect on Cyr61 expression and downregulates its expression in MCF-7 cells. Interestingly, most of these effects are not seen in aggressive breast cancer cells that do not express ER and express high levels of Cyr61, such as the MDA-MB-231 cells.

Our results are in agreement with our knowledge that Cyr61 promotes tumor growth, and that tumor-promoting agents have a positive impact on cells that express low levels of Cyr61,

such as the ER-positive breast cancer cells; however, these agents have no significant effect on cells that express high levels of Cyr61. Our findings provide further evidence that an increased level of Cyr61 correlates with breast cancer progression.